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Prognostic significance of epithelial—mesenchymal transition in malignant pleural mesothelioma☆,☆☆

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Abstract

Background: Epithelial-to-mesenchymal transition (EMT) is a morphologic transdifferentiation of carcinomas, conferring increased tumour invasiveness, but may also be applied to the epithelioid versus sarcomatoid histotype of malignant pleural mesothelioma (MPM). Herein, we correlated proteins of a putative MPM—EMT axis, including periostin, epidermal growth factor receptor (EGFR), integrin β1, phosphatase and tensin homologue (PTEN), integrin-linked kinase (ILK), p21 and p27, with clinico-pathologic parameters, in particular overall survival (OS). **Patients and Methods:** A retrospective cohort of 352 mostly untreated patients with MPM was investigated by immunohistochemistry of a tissue microarray. Protein expression intensities were semi-quantitatively scored from 0 to 3 in their respective compartments, including peritumoural stroma as well as tumour cell plasma membrane, cytoplasm or nucleus. Data were correlated with histotype and survival outcome. **Results:** A total of 32% of the tumours were diagnosed as epithelioid, 13% as sarcomatoid and 55% as biphasic histotype. High expression of membranous EGFR and integrin β1 as well as nuclear p27 correlated with the epithelioid and high expression of cytoplasmic tumoural and stromal periostin with the sarcomatoid histotype. The median survival time of the 128 patients with complete follow-up data was 11.7 months. Univariate survival analysis revealed age, epithelioid histotype and any therapy as prognosticators for better OS. High expression of cytoplasmic PTEN or ILK as well as high expression of nuclear p21 or p27 correlated with increased, whereas high expression of cytoplasmic periostin with decreased OS (all p values <0.05). Multivariate Cox regression revealed any treatment, low cytoplasmic periostin and high cytoplasmic PTEN as independent prognosticators for better OS. **Conclusion:** Activation of periostin-triggered EMT is associated with the sarcomatoid histotype and has an impact on shorter survival of MPM patients. Finally, only the high expression of PTEN and the low expression of cytosolic periostin could be shown to be independent prognostic factors for longer OS.

Keywords: Malignant pleural mesothelioma; Epithelial-to-mesenchymal transition; Periostin; PTEN; Tissue microarray; Histotype

1. Introduction

Despite multimodality treatment including neo-adjuvant chemotherapy, radical surgery and adjuvant radiotherapy, the prognosis of malignant pleural mesothelioma (MPM) is still limited. The aggressiveness of this tumour maybe explained by its partial fibroblastic phenotype in the context of epithelial-to-mesenchymal transition (EMT), conferring both high invasiveness and chemoresistance.

EMT is a developmental programme of foetal and tumoural cells, characterised by loss of cell adhesion, repression of E-cadherin expression and increased cell motility [1]. Concurrently, cells acquire expression of mesenchymal components and manifest a migratory phenotype. As tumours often mimic embryonic development, it is postulated that EMT is an important probably transient, event in the progression, invasion and metastasis of carcinomas and it may be a permanent feature in sarcomatoid carcinomas, which have a particularly dismal prognosis [2]. The inverse process is called mesenchymal—epithelial transition (MET) [3]. Apart from sarcomatoid carcinoma, which is rare in most organs, one might assume MPM to potentially be one of the best *in vivo* models for EMT—MET transdifferentiation, since it consists of two distinct histotypes — epithelioid and sarcomatoid — and the additional biphasic one may be considered a true intermediate, assembling cells with either of the two differentiations in close proximity. Importantly, mesothelial cells are of coelomic and therefore mesodermal origin, and thus may be viewed as cells having undergone at least partial EMT during embryogenesis.

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The molecular basis of EMT involves multiple changes in expression, distribution and/or function of proteins that include extracellular matrix and plasma membrane proteins such as periostin, vimentin, integrins, matrix metalloproteinases (MMPs) and cadherins [2,4]. With regard to signalling, several oncogenic pathways such as peptide growth factors, Src, Ras, Ets, Wnt/beta-catenin and Notch, can induce EMT [5]. Activation of the phosphatidylinositol-3 kinase (PI3K)/Akt axis seems to be a central feature of EMT, and this activation can be abolished by PTEN [6,7].

In the previous studies, we have demonstrated that cytosolic loss of PTEN is associated with decreased overall survival of MPM patients [8]. Furthermore, we have found the extracellular matrix protein periostin to be involved in EMT of non-small-cell lung cancer (NSCLC), whereby high stromal expression, in particular, was associated with decreased progression-free and overall survival [9].

In this study, we analysed the protein expression of individual members of a complete putative MPM–EMT signalling cascade, including the extracellular matrix and cell adhesion protein periostin, the two cross-talking plasma membrane receptors EGFR and integrin β1, the two associated adaptor and regulator proteins PTEN and ILK and the two downstream effectors of the Akt pathway, p21 and p27, in a larger, mostly untreated cohort of MPM (n = 352) by immunohistochemistry. We tested the null hypothesis that individual protein expression levels do not correlate with each other, or with clinico-pathologic parameters including histotype and OS.

2. Patients and methods

2.1. Patients and histological subtypes

Tissue specimens of 352 patients with MPMs, diagnosed between 1975 and 2004, were sent to the Zürich Pneumoconiosis research group for mineralogical assessment of dust exposure, in particular, asbestos, and later included in this study. The total cohort comprised 114 epithelioid, 192 biphasic and 46 sarcomatoid histotypes. A total of 77% of the specimens were derived from autopsy and 23% from biopsy specimens. Tissue was processed according to the guidelines of the Swiss Society of Pathology. All cases were entirely reviewed for histotype classification by two pathologists (S.T. and A.S.). Clinical data were retrospectively assessed from medical archives of the different hospitals and the local cancer registries.

2.2. TMA construction and immunohistochemistry

A set of three tissue microarrays (TMA) with quadruple punches per patient (total n = 1408) was accomplished with a custom-made, semi-automatic tissue arrayer (Beecher Instruments, Sun Prairie, WI, USA), as previously described [10]. Sections (4.5-μm thick) of TMA blocks were transferred to an adhesive-coated slide system (Instrumedics, Hackensack, NJ, USA) supporting the cohesion of 0.6-mm array elements on glass. De-paraffined sections were stained on either a Ventana (Ventana Medical Systems, Tucson, AZ, USA) or a Bond automat (Vision Biosystems, Melbourne, Australia), using the following primary antibodies: polyclonal rabbit anti-periostin (1:500, clone OSF-2, Biovendor Laboratory Medicine Inc., Modrice, Czech Republic), monoclonal anti-PTEN (1:200, clone 6H2.1, Dako, Glostrup, Denmark), monoclonal anti-EGFR (pre-diluted, clone 3C6, Ventana Medical System Inc., Tucson, AZ, USA), polyclonal anti-ILK1 (1:50, clone RB1668, Abgent, San Diego, USA), monoclonal anti-integrin β1 (1:800, clone 4B7R, Abcam, Cambridge, UK), polyclonal anti-p21 (1:50, clone SC-397, SCBT, Santa Cruz, CA, USA) and polyclonal anti-p27 (1:30, clone SC-528, SCBT, Santa Cruz, CA, USA). Detection with respective secondary antibodies was performed with Ultraview Amp (Ventana) or Refine-DAB (Vision Biosystems).

2.3. Data interpretation and statistical analysis

The intensity level of immunoreactivity of the EMT cascade proteins was scored semi-quantitatively: 0 (negative), 1 (weak), 2 (moderate) and 3 (strong) in stroma and tumour cells, including the plasma membrane, cytoplasm and nucleus as distinct compartments, by two observers (A.Sc./A.So.). A mesothelioma-cell-associated signal was considered if three or more cells were positive. A global sum score was created from the four cores and was dichotomised closest to the median into low and high. Clinical data were retrospectively assessed as completely as possible from the medical archives of different hospitals and the local cancer registry. The material of the patients came mainly from autopsies and there was no standardised therapy at this time period. Associations and correlations with clinico-pathologic parameters were examined by chi-squared and Kendall's tau-β tests, respectively, for both non-dichotomised as well as dichotomised parameters. OS was calculated by the Kaplan–Meier method and survival time differences were compared using the log-rank test. Significant parameters were further analysed by multivariate Cox proportional hazards regression models to test for independency. All analyses were carried out using the SPSS 16.0.1 software package (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Cohort description

The total cohort of 352 patients comprised 114 epithelioid, 46 sarcomatoid and 192 biphasic histotypes. Further clinical data could be retrieved from 206 of these 352 patients (59%), who were mainly males (94%). The median age was 62 years. Asbestos exposure was known in 97 patients (47%). Disease was located in 52% of the patients on the right, 36% on the left and 3% on both sides. As tumour stage was not documentable in most cases, a retrospective staging was performed for 102 patients, based on the surgical pathology reports according to the IMIG staging system [11], stage T4 being predominant with 71%. Both regional and mediastinal lymph nodes as well as distant metastases were present in 90% of the patients. Only 30% of the patients received therapy, 70% were treatment-naïve. Treatment was surgical for 67 patients and comprised 26 extrapleural pneumonectomies, 16 pleurectomies and 25 palliative procedures, such

Table 1
Survival data for patients with available complete follow-up data ($n = 128$).

	$n = 128$[a]	%	Univariate				Multivariate			
			Med OS	95% CI		p-value	HR	95% CI		p-value
				Lower	Upper		Exp (B)	Lower	Upper	
Age										
<62 years	66	52	12.6	8.4	16.9					
>62 years	62	48	10.9	6.7	15.2	0.029				
Gender										
Male	120	94	11.7	9.6	13.9					
Female	8	6	11.6	8.1	15.2	0.077				
Treatment										
No	71	57	9.8	7.1	12.4					
Any	54	43	14.2	9.5	18.9	0.002	0.644	0.433	0.957	0.029
Histotype										
Epithelioid	38	30	12.7	8.4	17.0					
Sarcomatoid	17	13	6.5	4.9	8.1					
Biphasic	73	57	13	11.2	14.8	0.009				
Periostin Stroma										
Low	73	57	12.7	10.6	14.8					
High	55	43	11.1	6.6	15.7	0.171				
Periostin Tu-Cyto										
Low	70	55	14	9.7	18.4					
High	58	45	9.5	5.9	13.1	<0.001	1.824	1.249	2.665	0.002
EGFR Tu-Mem										
Low	70	55	11.6	9.5	13.7					
High	58	45	11.7	8.5	15.0	0.296				
Integrin β1 Stroma										
Low	69	54	10.7	9.2	12.3					
High	58	46	13	12.1	14.0	0.109				
Integrin β1 Tu-Mem										
Low	65	51	11.7	8.9	14.5					
High	62	49	11.5	8.5	14.5	0.902				
ILK Tu-Cyto										
Low	62	48	10.7	7.8	13.7					
High	66	52	13.2	11.1	15.3	0.022				
PTEN Tu-Cyto										
Low	79	62	10.3	8.3	12.3					
High	48	38	15.5	3.8	27.2	<0.001	0.560	0.367	0.854	0.007
p21 Tu-Nuc										
Low	66	52	10.3	8.2	12.3					
High	62	48	13.2	12.2	14.2	0.006				
p27 Tu-Nuc										
Low	62	49	10.7	8.3	13.1					
High	65	51	13.2	12.1	14.3	0.023				

Kaplan–Meier analysis with log rank test.
[a] Number of patients varying, dependent on achievable data, $n = 127$ for integrin β1 membrane and stroma, PTEN and p27, $n = 125$ for treatment.

as talc pleurodesis or tumour debulking. A total of eight patients received only chemotherapy in different combinations, mostly platinum-based. Four patients received radiation therapy. The median overall survival of the 128 patients with complete follow-up data (Table 1) was 11.7 months (95% confidence interval (CI): 10.0–13.6).

3.2. Protein expression patterns

In the extracellular matrix of the peritumoural stroma, periostin and integrin β1 protein expressions were found in 99% of the tumours (any core with any positivity 1–3) in the form of a fibrillary staining (Fig. 1). In the tumour cells, cytosolic expression of periostin was detectable in 96%, of PTEN in 38% and of ILK in 49%. Plasma membrane expression of EGFR was observed in 86%, of integrin β1 in 99%; nuclear expression of p27 in 68% and of p21 in 47% of the tumours. Table 2 shows the frequency distribution of protein expression intensities per individual core (0 = negative, 1 = weak, 2 = moderate and 3 = strong).

3.3. Correlation with clinico-pathologic parameters

An association of any marker expression and an asbestos exposure could not be found in this retrospectively collected data. Detailed histotype analyses per TMA core revealed that

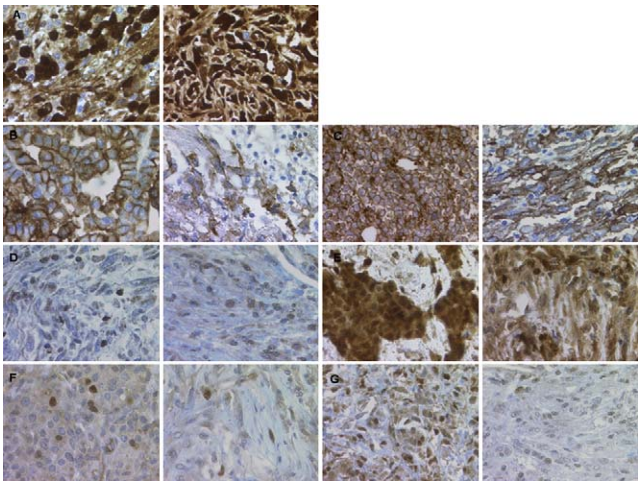

Fig. 1. Examples of EMT protein marker expression in a patient each with epithelioid (left panel) and sarcomatoid (right panel) mesothelioma, respectively (magnification ×400): (A) tumour and stroma cells showing a variable expression of periostin in both the epithelioid and sarcomatoid histotype. (B) Membranous EGFR staining. (C) Integrin β1 in comparison to EGFR, also with strong membranous expression. (D) Strong staining of ILK in the cytoplasm, while nuclei remain unstained. (E) PTEN localised to the cytoplasm. (F) p21 presenting a variably strong nuclear expression. (G) p27 presenting a variably strong nuclear expression.

Table 2
Frequency distribution of protein expression intensities (0 = no expression, 1 = low, 2 = moderate, 3 = strong) of the EMT markers per the individual cores in percent.

	0 (%)	1 (%)	2 (%)	3 (%)
Periostin Stro	3.6	26.6	22.1	47.7
Periostin Tu-Cyto	12.0	24.1	21.7	42.2
EGFR Tu-Mem	23.5	40.0	28.7	7.8
Integrin β1 Stro	3.0	32.1	38.8	26.1
Integrin β1 Tu-Mem	8.2	34.6	30.5	26.7
ILK Tu-Cyto	62.6	25.3	10.3	1.8
PTEN Tu-Cyto	69.5	13.4	6.3	10.7
p21 Tu-Nuc	70.6	25.4	3.6	0.4
p27 Tu-Nuc	44.5	43.2	9.3	3.0

high expression of both stromal as well as cytoplasmic periostin were associated with the sarcomatoid histotype. By contrast, high membranous EGFR and integrin β1 as well as high nuclear p27 levels were associated with the epithelioid histotype. Correlation among markers showed that most of them (EGFR, integrin β1, ILK, PTEN, p21 and p27) were positively correlated with each other except periostin, which also showed negative correlations. High expression of stromal periostin was positively correlated with high stromal integrin β1, but negatively with high EGFR, membranous integrin β1, PTEN and p27 (Table 3).

3.4. Survival analysis

Univariate survival analysis revealed age, histotype and therapy as OS prognosticators for the 128 patients with complete follow-up data (Table 1). Further, high cytoplasmic periostin was associated with decreased OS, whereas high cytoplasmic PTEN and ILK and as well as high nuclear p21 and p27 were inversely correlated with better OS. By contrast, the protein expression level of EGFR at the tumour cell plasma membrane did not have significant survival impact. All significant parameters of the univariate analysis were introduced into a Cox proportional hazard ratio model in order to test for independency (Table 1). Treatment (hazard ratio (HR) 0.644; 95% CI: 0.433–0.957, $p = 0.029$) as well as low cytoplasmic periostin (HR 1.824; 95% CI 1.249–2.665; $p = 0.002$) and a high cytoplasmic PTEN (HR 0.560; 95% CI 0.367–0.854; $p = 0.007$) were maintained as independent prognosticators for better overall survival (Fig. 2).

4. Discussion

In this large bank of formalin-fixed and paraffin-embedded tissue of 352 MPM patients, mainly treatment-naive patients were screened for the expression of different markers belonging to a putative EMT axis. Low expression of periostin and high expression of PTEN in the tumour cell cytoplasm were independent factors for better OS.

In this study, we focussed on the periostin–integrin axis [12], which is known to cross talk with the EGFR pathway at the plasma membrane level (Fig. 3). We could find an association of high cytoplasmic periostin with the sarcomatoid histotype and with decreased OS; thus, assessment of this protein might help identify the sarcomatoid histotype [10]. It is well conceivable that both EMT of mesothelioma cells as well as increased peritumoural fibrosis are important factors for high invasiveness and chemoresistance of MPM, respectively.

Oncogenic EGFR is overexpressed in many tumours, including MPM [13], but without impact on survival [14]. Herein, the same result was found. Furthermore, clinical studies have shown that treatment with tyrosine kinase inhibitors had no impact on MPM patients' survival. EGFR is influenced by asbestos as shown in *in vitro* experiments and leads to an activation of the Erk pathway acting on cell survival and proliferation [14].

Integrin β1 is a transmembrane receptor not only for extracellular matrix proteins such as fibronectin, laminin and collagen, but also for periostin [15]. As a result of binding, various cellular responses are observed, including alterations in survival, proliferation, spreading and migration, gene transcription and differentiation [16]. Downstream signalling with cytoplasmic stabilisation of the cell cycle regulators p21 (WAF/CIP1) and p27 (KIP1) by Akt is modified by PTEN and ILK [17]. Both markers correlated with longer survival in our analysis, PTEN with even independent prognostic value, underscoring its function as tumour suppressor. PTEN is located underneath the plasma membrane and has important regulatory functions at this position on signal transduction from the extracellular matrix to the tumour cell nucleus, mainly by action on Akt [18,19]. Akt is assumed to be the most important player in this axis and was proven to be involved in proliferation of different tumours, including MPM. *In vitro*, reduction of mesothelioma cell proliferation and motility was achieved by targeting the PI3K/Akt pathway with PI3K inhibitors [20]. Akt staining was performed with different commercially available antibodies but was not reproducible, so that the results were omitted from analysis. ILK is a protein kinase inducing a range of signalling pathways after integrin

Table 3
Correlation of EMT marker expression with histotype and among each other on individual cores (n = 1408).

	Periostin		EGFR	Integrin β1		ILK	PTEN	p21	p27
	Stroma	Tu-Cyto	Tu-Mem	Stroma	Tu-Mem	Tu-Cyto	Tu-Cyto	Tu-Nuk	Tu-Nuk
Individual									
Histotype									
Ep-Sa-Bi									
tau	0.222	0.110	−0.138	0.013	−0.063	0.072	0.070	0.106	0.008
p	<0.001	<0.001	<0.001	0.616	0.013	0.005	0.007	<0.001	0.754
Ep-Sa									
tau	0.296	0.211	−0.426	0.012	−0.096	0.008	−0.015	0.039	−0.085
p	<0.001	<0.001	<0.001	0.698	0.002	0.797	0.634	0.206	0.006
Periostin									
Stroma									
tau		0.539	−0.105	0.112	−0.126	0.011	−0.070	0.036	−0.008
p		<0.001	<0.001	<0.001	<0.001	0.690	0.006	0.180	0.753
Tu-Cyto									
tau			−0.009	0.130	0.106	0.000	−0.151	0.009	0.020
p			0.742	<0.001	<0.001	0.996	<0.001	0.727	0.453
EGFR									
Tu-Mem									
tau				0.076	0.315	0.176	0.169	0.191	0.270
p				0.004	<0.001	<0.001	<0.001	<0.001	<0.001
Integrin β1									
Stroma									
tau					0.189	0.038	0.105	0.117	0.124
p					<0.001	0.151	<0.001	<0.001	<0.001
Tu-Mem									
tau						0.069	0.079	0.197	0.218
p						0.010	0.002	<0.001	<0.001
ILK									
Tu-Cyto									
tau							0.414	0.321	0.346
p							<0.001	<0.001	<0.001
PTEN									
Tu-Cyto									
tau								0.430	0.470
p								<0.001	<0.001
p21									
Tu-Nuk									
tau									0.494
p									<0.001
p27									
Tu-Nuk									
tau									
p									

Kendall's tau-β test (tau = correlation coefficient, p = p-value).

activation and was found to be overexpressed in MPM by Watzka et al. [21], thereby correlating with higher patient's age. Herein, ILK was expressed in 49% of MPM with any positivity, and had impact, although not independent, on OS.

The two cyclin-dependent kinase inhibitors p21 and p27 are downstream markers in the Akt pathway. Nuclear import of p21 and p27 is thereby supposed to be increased, leading to growth control. More recently, both proteins have been implicated in EMT through transcription factors such as Snail or Twist [22,23]. In MPM, high expression of p27 correlated with better patient's survival [24], a finding which is corroborated by our results, although without independent prognostic value.

For better stratification of patients, precise prognostic and diagnostic markers to select MPM patients who could benefit from treatment are required. According to the results of this study, patients with high cytoplasmic periostin combined to decreased cytoplasmic PTEN may be defined as high-risk group for poor prognosis. One of the disadvantages of the study is the retrospective nature of

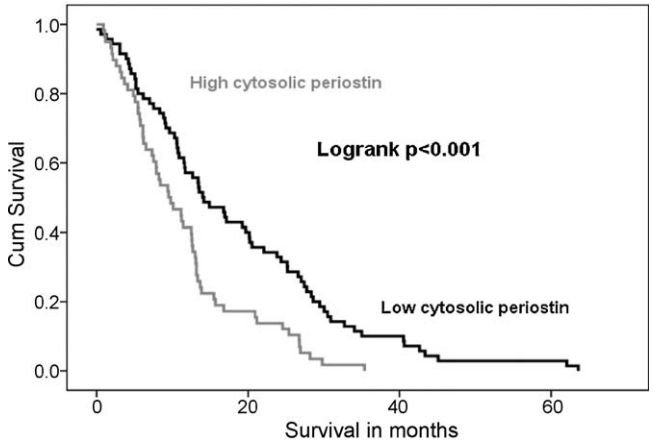

Fig. 2. Overall survival with dichotomised cytoplasmic periostin expression. Low expression (black curve, median overall survival 12.7 months (95% CI 9.7—18.4 months)) and high expression (gray curve, median overall survival 9.5 months (95% CI 5.9—13.1)).

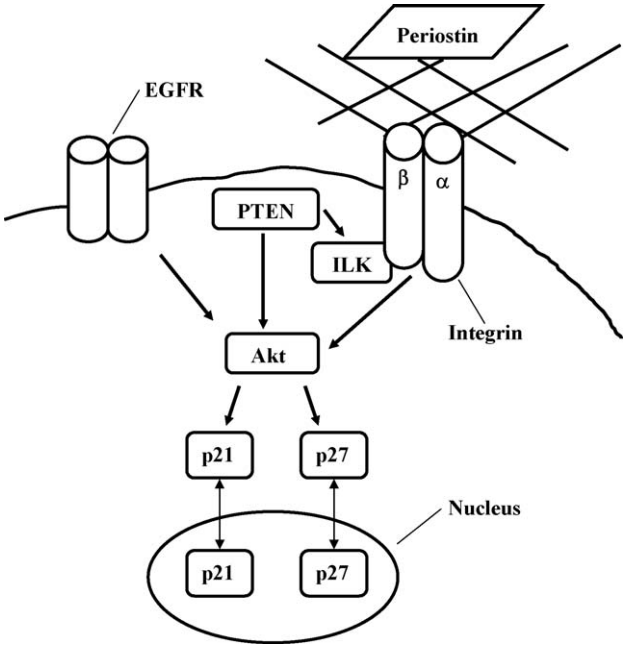

Fig. 3. Schematic picture of the proposed EMTaxis onto p21 and p27, triggered by periostin.

the clinical data. Therefore, correlation of protein marker expression with tumour stage and therapy was limited. It is planned to evaluate the prognostic impact of these markers in selected patients who underwent standardised multimodality therapy, including neo-adjuvant platinum-based chemotherapy, extrapleural pleuro-pneumonectomy and radiotherapy [25].

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